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Impacts of elevated temperature and CO_2 concentration on growth and phenolics in the sexually dimorphic *Populus tremula* (L.)☆

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ABSTRACT

Future climatic changes may alter the balance between the sexes of dioecious species due to differential effects on resource allocation to growth and defense. Our purpose was to study the impacts of elevated temperature and CO_2 concentration on the relative changes in growth and phenolics accumulation in stem bark of the dioecious *Populus tremula*, a keystone species for boreal forest biodiversity and one that is browsed by many mammalian herbivores. In a greenhouse experiment, four female and four male genotypes of *P. tremula* were grown under single and combined treatments of elevated temperature (1.5 °C on average) and CO_2 concentration (720 ppm) for one growing season. Elevated temperature increased the height, diameter, leaf and stem biomass, and in addition decreased the concentration of phenolics including salicylates, flavonoids, phenolic acids, salireposide and lignan. Elevated CO_2 concentration, on the other hand, reduced the height growth (other growth parameters were unchanged) and increased the concentration of phenolics, especially salicylates and phenolic acids. In the combined treatment, *P. tremula* tended to grow more, but elevated temperature counteracted the effect of elevated CO_2 concentration on phenolics accumulation. Although statistically not significant, males tended to have greater growth and a lower level of phenolics than females. The smaller sexual differences were also not strongly affected by climatic factors. Under future elevated temperature and CO_2 concentration, both sexes of *P. tremula* will probably grow more and possibly accumulate lower levels of phenolics, but intersexual differences in growth and phenolics accumulation may be more pronounced after sexual maturation.

1. Introduction

Climate change is likely to continue over the current century, even if the signed treaties are implemented (IPCC, 2013). Increasing anthropogenic CO_2 emission to the atmosphere is mainly responsible for climate change, which triggers other environmental factors, for example the rise of global air temperature. If the emission of CO_2 continues to increase at the current pace, it will reach 540–970 ppm by the end of this century and will lead to a rise in air temperature of 2–5 °C (IPCC, 2013; Gherlenda et al., 2015). Relative to the global mean, temperature elevation is anticipated to be even higher in the northern hemisphere due to the diminishing snow cover (Peng et al., 2013). Both CO_2 and temperature are limiting factors for plant growth, development, reproduction, and primary and secondary metabolism, possibly affecting plant distribution, production and abundance in coming years.

Under optimal conditions, CO_2-fertilized plants exhibit greater growth initially as a result of increasing photosynthesis and decreasing transpiration (Meng et al., 2013). With time, the increasing growth rate is reduced due to photosynthetic down-regulation resulting from the declining carbon demand and low nutrient availability (Peñuelas and Estiarte, 1998; Usami et al., 2001). In addition to growth, plants need CO_2 to produce carbon-based secondary metabolites (CBSM). This may result in a trade-off between growth and secondary metabolism. Since elevated CO_2 with limited nutrient supply weakens the utilization of carbon to growth (carbon sink), surplus carbon is then allocated to CBSM (e.g. Peñuelas and Estiarte, 1998). However, empirical studies have provided results both in favor of and against this hypothesis (Hamilton et al., 2001; Lindroth, 2012). Moreover, the effects of elevated CO_2 on secondary metabolites can vary depending on the compound in question. For example, in *Betula pendula,* elevated CO_2 concentration reduced the flavonol glycosides, while the concentrations of total phenolics, and individual condensed tannins and (+)-catechin increased (Kuokkanen et al., 2003).

Since tree growth in the northern hemisphere is temperature limited (Way and Oren, 2010), growth may increase until the temperature

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reaches its optimum. Accordingly, it has been found that a 2 °C increase in temperature significantly enhances the height, basal diameter and aboveground biomass in *Salix myrsinifolia* (Veteli et al., 2002; Nybakken et al., 2012), and the photosynthesis, specific leaf area, height, basal diameter and aboveground biomass in *Populus tremula* (Randriamanana et al., 2015). Experimental studies show that elevated temperature increases carbon sink, which is contrary to the effects of elevated CO_2 concentration. Increasing carbon sink makes less carbon available for allocation to CBSM. As a result, plants grown at an elevated temperature usually contain lower concentrations of CBSM in their tissues (Kuokkanen et al., 2001; Veteli et al., 2002; Randriamanana et al., 2015). However, there are also studies where elevated temperature have been shown to increase the concentration of CBSM (e.g. Goh et al., 2016; Zhao et al., 2016).

Because increase in CO_2 and temperature are concomitant, and plants respond to both of them, one factor can intensify or weaken the effect of another (Nybakken and Julkunen-Tiitto, 2013; Nissinen et al., 2016). Accordingly, an increase in CO_2 concentration enhances the optimum temperature for light-saturated photosynthesis, which can be seen as greater biomass accumulation in combined than in single CO_2 and temperature treatments (Long, 1991; Usami et al., 2001). While plant growth and CBSM are relative to each other, the combined effect of temperature and CO_2 concentration on growth performance is presume to alter the concentration of CBSM in plant tissues. For example, in three boreal broad leaf trees (*B. pendula, B pubescens* and *S. myrsinifolia*), elevated temperature diminished the effects of elevated CO_2 in a broad range of CBSM including salicylates, flavonoids and phenolic acids, although the magnitude of the interaction varied across the species (Veteli et al., 2007). In a meta-analysis, Zvereva and Kozlov (2006) concluded with similar effects over a wide range of plant functional groups.

While many studies have been conducted on CBSM in leaves in response to elevated temperature and CO_2 concentration, relatively little attention has been paid to CBSM in stem, particularly in stem bark. CBSM in stem bark, however, have a variety of ecological functions, including slowing the spread of pathogens, deterring herbivores from feeding, protection against UV radiation and providing strength to tissues to protect them against mechanical injury (Tahvanainen et al., 1991; Smith, 1997; Kuokkanen et al., 2001; Heiska et al., 2007; Lindroth et al., 2007; Julkunen-Tiitto et al., 2015). In several species, the quality and quantity of CBSM in stem bark seem to be very important. For example, bark and twigs of *Populus, Betula* and *Salix* species are a major source of food for many mammalian herbivores during winter (Kuokkanen et al., 2001; Lindroth et al., 2007; Boeckler et al., 2011). For this reason, relative changes in growth and CBSM in stem bark in response to elevated CO_2 and temperature may have serious consequences for the prominence of these species in future climatic conditions. Among the *Populus* species, *P. tremula* is particularly important in boreal forest biodiversity. It is a keystone species and more than 200 other species depend on it as a source of food or as a habitat in Finland alone (Siitonen, 1999; Kouki et al., 2004).

In the case of dioecious species, females and males differ in their priorities for the allocation of resources to different functions. According to Ågren et al., 1999, females are innately more defensive, and they may allocate more resources to the accumulation of secondary metabolites and reproduction than to an increase in plant size. Males, on the other hand, prefer to invest more in growth than in defense and reproduction (Ågren et al., 1999; Nybakken et al., 2013; Maja et al., 2016). If plants are too young for sexual reproduction, a trade-off may only occur between growth and defense. Under the predicted elevated temperature and CO_2 concentrations, females and males may vary in their resource distribution, which in turn might affect their productivity and population structure. For instance, in response to elevated temperature and CO_2 concentrations, males of *P. cathayana* benefited more from elevated CO_2 by increasing leaf size and biomass accumulation than did females (Zhao et al., 2012). Jones et al. (1999) also measured greater carbon assimilation in male individuals of *S. arctia* under the combined effects of elevated temperature and CO_2. Therefore, if elevated CO_2 and temperature further stimulate the growth of male population, this may reduce their accumulation of CBSM, and it is supposed that males will face more damages due to biotic factors (such as herbivory) in coming years.

In this study, we particularly seek answers to how males and females of *P. tremula* plants do balance between growth and CBSM synthesis in stem bark under the single and combined effects of elevated temperature and CO_2 concentration. We hypothesized that (1) elevated temperature will increase the growth and decrease the concentration of CBSM, (2) elevated CO_2 concentration will have no effect on growth, since the initial growth stimulation effect of elevated CO_2 concentration is not usually sustained through time in broad-leaved trees (Tjoelker et al., 1998; Vu et al., 2002), and surplus carbon will thus increase the concentration of CBSM, (3) in the combined treatment, elevated temperature and CO_2concentration may cancel out each other's effect, and (4) inherently higher growth of males and higher phenolic concentration in females may be further increased by elevated temperature and CO_2 concentration, respectively.

2. Materials and methods

2.1. Plant material

This experiment was carried out with micropropagated European aspen (*Populus. tremula* L.) plantlets. Eight separate clonal lines were produced from the axillary buds of about 30–40 years old *P. tremula* trees from different sites in Eastern and Southern Finland (Table S1). The mother trees were selected from different locations to ensure genotypic differences among them. The plantlets were regenerated in glass jars (about 3 to 4 weeks) *in vitro* on woody plant medium (WPM) comprised of agar (8.5 g l^{-1}) and indole butyric acid (5 mg l^{-1}) under a controlled environment of temperature (23 ± 0.1 °C), photoperiod (18/6 h light/dark) and photosynthetically active radiation (70 µmol m^{-2} s^{-1} at 400–750 nm) for root development.

Micropropagated tiny plantlets from the glass jars were transferred to plastic trays (size: 40 × 40 × 9 cm) and raised in the greenhouse (Joensuu, Eastern Finland) for acclimation from April 13 to May 18, 2015. Each plastic tray had 30 cells filled with 70% commercial peat and 30% vermiculite, with one plantlet in each cell. The environmental conditions in the greenhouse were maintained at temperature of 20 ± 3 °C, air relative humidity of 70% and photoperiod of 18/6 h light/dark. The plantlets were transferred to the greenhouse experiment on May 19 and replanted in 1L plastic pots on May 20, 2015. The pots were filled with the same proportion of commercial peat and vermiculite as during the acclimation period. In each greenhouse room, 8 plantlets (one/genotype) were placed randomly. The pots were moved around every week in order to provide identical growth conditions. The plantlets were watered regularly. They were fertilized twice (on June 4 and July 6) with N: P: K = 19.0%: 4.4%: 20.2% and with other elements as follows: (Mg) 1.2%, (MgO) 2.0%, (B) 0.03%, (Co) 0.001%, (Cu) as the EDTA chelate 0.008%, (Fe) as the EDTA chelate 0.17%, (Mn) as the EDTA chelate 0.08%, (Mo) 0.005%, and (Zn) as the EDTA chelate 0.012%. Each plant received a dose of 250 ml fertilizer solution each time (for details see Kosonen et al., 2012).

2.2. Experimental design

The experiment was conducted in greenhouses at the Mekrijärvi Research Station (62°47′N, 30°58′E, 145 m a.s.l., University of Eastern Finland) from May 20 to August 5, 2015. Sixteen greenhouse rooms were randomly assigned including four control (C), four elevated temperature (T), four elevated CO_2concentration (CO_2) and four combined (CO_2 + T) treatments (n = 4). Ambient temperature was achieved by following the outside air temperature through a modulated system, whereas elevated temperature was set to 2 °C above this ambient level.

Based on the values recorded at every minute, the achieved elevated temperature (17.6 ± 2.6 °C) was on average 1.5 °C higher than temperature in the ambient chambers (16.1 ± 2.7 °C) for the whole experimental period (Fig. S1a). PT1000 temperature sensors (Czech Republic) were used to follow the temperature (accuracy ± 0.3%) in the measuring range of −40 to +60 °C. Ambient and elevated CO_2 concentrations were set at 400 and 720 ppm, respectively (Fig. S1b). We also followed the relative humidity (Rh 0-100%) in all the rooms and maintained it at a set point of 60% (TRH-302A, accuracy ± 3%; Nokeval Ltd, Finland).There was no UVB treatment, but the ambient level of ultraviolet-B radiation (UVB) was maintained in all the rooms by means of UV lamps (1.2 m long, UVB-313, Q-panel Co, Cleveland, OH) which constantly followed outside UVB conditions. The technical details of the chamber regulations and maintenance of treatment properties are described in Zhou et al. (2012).

2.3. Growth measurements and sampling

The height growth and basal diameter of all the plantlets were measured six times at about two-week intervals. The height was measured from the root collar to the tip of the longest shoot (± 0.5 cm) with measuring sticks while the diameter was measured 1 cm above the root collar (± 0.01 mm) with vernier calipers.

All the plantlets were harvested on August 4–5, 2015. A 10 cm fresh stem section from the part where the first mature leaves appeared for each plantlet were collected for secondary chemistry analyses. The stem section was divided into two longitudinal halves using a sharp knife after removing the leaves to ensure proper drying. They were placed in small paper bags with drying media and air-dried in a drying room at 10% relative humidity and 22 °C temperature. The dry samples were kept in the freezer (−20 °C) until extractions.

For biomass measurement, the rest of the plant was collected in paper bags and dried at room temperature in the greenhouses for about a month (August 6 to September 7, 2015). After drying, leaf and stem biomass were weighted separately.

2.4. Analyses of stem bark phenolics

The analysis of stem bark phenolics was performed according to Nybakken et al. (2012). Before extraction, bark (inner and outer) was peeled from the xylem section and cut into tiny pieces. About 20 mg of bark was then weighed into a vial with three stainless steel balls, and powdered in a Precellys[24] (Bertin Technologies, Montigny-le-Bretonneux, France) homogenizer. 600 µl of pure ice-cold methanol (HPLC grade) was added to the powder and homogenized for 25 s at 5500 rpm. Thereafter, the samples were incubated in an ice-bath for 15 min. The samples were later re-homogenized before centrifuging (Eppendorf® centrifuge 5415 R, Eppendorf, Hamburg, Germany) at 13000 rpm for 3 min. The supernatants were collected in 6 ml glass vials. This process of extraction was repeated three more times, but the samples were then incubated in the ice-bath for 5 min. The combined supernatants were dried at 45 °C using a vacuum centrifuge (Eppendorf 270 concentrator, Hamburg, Germany). The dried extracts were kept in a freezer (−20 °C) until high-performance liquid chromatography (HPLC) was performed.

For a HPLC analysis, the dried extracts were re-dissolved in 600 µl of methanol-water (1:1) and transferred to eppendorf-vials. They were then centrifuged at 13000 rpm for 3 min. The supernatants were separated into HPLC-vials and 7 µl of each sample was injected for analysis. The HPLC (Series 1100, Agilent, Germany) used for separation was equipped with a binary pump (G1312A), an ALS autosampler (G1329A), a vacuum degasser (G1322A), a column compartment (G1316A) with a reverse-phase column (Zorbax SB-C18, 4.6 × 75 mm, particle size 3.5 µm, Agilent) and a diode array detector (G1315B).The mobile phase was composed of two eluents as follows: eluent A (1.5% tetrahydrofuran and 0.25% orthophosphoric acid in Milli-Q ultrapure water) and eluent B (100% MeoH) with a flow rate of 2 ml min^{-1}. The injector and column temperature were maintained at 22 °C and 30 °C, respectively. The compounds were identified as in Randriamanana et al. (2015). Quantification of compounds at 220, 270 and 320 nm was based on the standards as follows: salicinfor salicin, diglucoside of salicyl alcohol and lignan; salicortin for salicortin, HCH- salicortin, salicortin derivative and disalicortin; (±)-catechin for (+)-catechin and gallocatechin; chlorogenic acid for chlorogenic acid derivative 1 and 2; salireposide for salireposide; *p*-OH-cinnamic acid for *p*-OH-cinnamic acid glucoside, *p*-OH-cinnamic acid derivative, cinnamoyl salicortin, cinnamoyl salicortin derivative 1, 2 and 3; cinnamic acid derivatives for cinnamic acid derivative 1, tremulacin for tremulacin; myricetin 3-rhamnoside for myricetin galactoside, myricetin derivative and myricetin 3-glucoside. Arbutin (Sigma Chemical Company, St. Louis, USA) was used as an internal standard to measure the extraction efficiency. The internal standard recovery rate was 88.55 ± 2.56% (n = 8). Therefore, concentrations of the compounds were converted to 100% to recover the loss of 11.45% during extraction.

2.5. Statistical analyses

The effects of CO_2, temperature and sex and their interactions on the growth parameters and secondary chemistry were examined by a linear mixed-effects model using IBM SPSS Statistics for Windows (Version 21.0. Armonk, NY: IBM Corp). In analyzing height and diameter data, CO_2, temperature and sex were used as fixed factors, and clone as a random factor. Moreover, measurement date was set as a repeated variable, and the first measurement values of the parameters were set as covariates. In the case of biomass and bark secondary chemistry, CO_2, temperature and sex were use as fixed factors, and clone as a random factor. The normality of residuals was checked, and if needed, the data were transformed to meet the assumptions (see Table 3).

2.6. Graphic vector analysis

A graphic vector analysis (GVA) was performed to illustrate whether the changes in phenolic concentrations are a result of shifting in secondary chemical synthesis or shifting in biomass production under the treatments of elevated CO_2 and temperature. In a GVA, the relative values of phenolic content, phenolic concentration and plant biomass are plotted on the x, y and z axis, respectively. The center of the graph represents the reference point (control) where x = y = z = 100. Relative values were calculated according to the following formula: (treated mean/control mean) x 100. Excess synthesis increases both the content and concentration of a phenolic compound whereas reduced synthesis causes a decrease in both of them. If the concentration is increased along with the decrease in content, we find a concentration effect. On the other hand, a dilution effect is the result of decreasing concentration along with an increase in content. For further clarification, please check Haase and Rose (1995) and Koricheva (1999).

3. Results

3.1. Growth

Elevated temperature, CO_2 concentration and sex affected the height growth of *P. tremula* seedlings (Table 1). Elevated temperature increased the mean height growth by 54% compared to the control plants, while elevated CO_2 concentration reduced the height by 11% (Fig. 1a). When elevated temperature was combined with elevated CO_2 concentration, the height increment remained 41% greater than controls (Fig. 1a). In males, overall height growth was 18% greater than in their female counterparts (Fig. 1a). However, height growth increase under elevated temperature was greater in females than males and resulted in 85% and 33% increase, respectively (Fig. 1a). Moreover, sex-dependent variation in height growth under temperature further varied over time which resulted in the significant T x Sex x Time interaction

Table 1
F-values and degrees of freedom (df) obtained from the linear mixed model analysis of the effects of fixed factors and their combinations on the height and diameter of *P. tremula*. Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Arrows indicate the increase or decrease of the parameters. The letters "f" and "m" indicate the significant increment in females and males, respectively.

Factor	Height			Diameter		
	F	*Numerator df*	*Denominator df*	*F*	*Numerator df*	*Denominator df*
T	284.051***↑	1	316.810	72.846***↑	1	416.418
CO_2	17.989***↓	1	316.810	23.455***↑	1	416.418
Sex	6.812*m	1	7.232	1.047	1	6.980
Time	699.920***	5	160.809	2405.270***	5	203.440
T x CO_2	0.003	1	316.810	3.915*↑	1	416.418
T x Sex	11.444***f	1	316.810	4.316*f	1	416.418
T x Time	49.882***	5	160.809	11.602***	5	203.440
CO_2 x Sex	1.697	1	316.810	4.735*f	1	416.418
CO_2 x Time	3.405**	5	160.809	1.354	5	203.440
Sex x Time	8.806***	5	160.809	2.691*	5	203.440
T x CO_2 x Sex	0.182	1	316.810	0.726	1	416.418
T x CO_2 x Time	0.086	5	160.809	1.429	5	203.440
T x Sex x Time	2.457*	5	160.809	0.632	5	203.440
CO_2 x Sex x Time	0.304	5	160.809	1.809	5	203.440
T x CO_2 x Sex x Time	0.030	5	160.809	0.354	5	203.440

(Table 1, Fig. 1a).

Diameter growth increased under elevated temperature (Table 1). The diameter increment was 6% greater under elevated temperature compared with that of the control plants (Fig. 1b). In the combined treatment, diameter growth was 16% greater than in the control plants (Fig. 1b). No main effect of sex was detected, but there was a significant T x Sex interaction (Table 1) meaning that diameter increment was greater in females than in males in response to elevated temperature (9% vs. 2%) (Fig. 1b).

Elevated temperature also affected leaf, stem and total aboveground biomass (Table 2). Elevated temperature increased leaf biomass by 39% (Fig. 2a), stem biomass by 49% (Fig. 2b) and total aboveground biomass by 44% (Fig. 2a, b) when compared to the control individuals. Under CO_2 + T, leaf, stem and total aboveground biomass increased by 48, 56 and 52%, respectively (Fig. 2a, b). There were no effects of elevated CO_2 concentration alone on leaf, stem and total aboveground biomass. Although not statistically significant, male individuals had greater leaf (18%, Fig. 2a), stem (15%, Fig. 2b) and total aboveground biomass (16%, Fig. 2a, b) as compared to their female counterparts when averaged over all the treatments.

3.2. Stem bark phenolics

A total of 23 low molecular weight phenolic compounds were identified and quantified in the *P. tremula* stem bark samples (Table 3). Salicylates alone constituted 74% of the total concentration followed by salireposide (13%), flavonoids (5%) and lignan (4%). Phenolic acids was the smallest group of the identified phenolic compounds in terms of concentration (3%). In general, elevated temperature reduced the total concentration of phenolics while it increased under elevated CO_2 concentration (Table 3, Fig. 3f). However, some of the individual compounds responded differently. For example, disalicortin, cinnamoyl salicortin, and cinnamic acid derivative 1 responded to neither of the treatments (Table 3). Diglucoside of salicyl alcohol, HCH salicortin and gallocatechin increased in response to elevated temperature (Table 3). As a whole, female individuals had higher concentrations of total phenolic compounds in comparison with their male counterparts, but the difference was not statistically significant (Table 3, Fig. 3f).

Among the 11 identified salicylates, tremulacin (47%) and salicortin (33%) were the compounds with highest concentration. The total concentration of salicylates decreased by 22% under elevated temperature, while under elevated CO_2 concentration it increased by 6% in comparison with the control treatment (Fig. 3a). In the combined treatment, elevated CO_2concentration alleviated the effect of elevated temperature in lowering the total concentration of salicylates (Fig. 3a). Female plants had a 12% greater concentration of salicylates than did their male counterparts. GVA revealed that the total concentration of salicylates was reduced under temperature in both males and females as a result of a dilution effect (Fig. 4a, b). However, under elevated CO_2 concentration, excess synthesis occurred in male plants (Fig. 4b).

There were five phenolic compounds belonging to the flavonoid group. Proportionally, (+)-catechin (33%) and a myricetin derivative (33%) were the most prominent among the flavonoids. The total concentration of flavonoids was reduced in response to elevated temperature (28%), due to a dilution effect in females (Fig. 4c) and in males, due to reduced synthesis (Fig. 4d). Elevated CO_2concentration alone did not affect the total concentration of flavonoids, but there was a significant effect of CO_2 x Sex interaction (Fig. 3b, Table 3). Females had a 19% higher total concentration of flavonoids in comparison with males (Fig. 3b), and in females, elevated CO_2 concentration reduced the synthesis (Fig. 4c), where as in males, it was increased (Fig. 4d).

A total of 5 compounds were identified as phenolic acids. A *p*-OH-cinnamic acid derivative was the most concentrated among them, contributing to 44% of the total concentration of phenolic acids. Elevated temperature reduced the total concentration of phenolic acids by 55% when compared with the control plants (Fig. 3c). Elevated CO_2 concentration increased the total concentration of phenolic acids, and reduced the magnitude of the temperature effect under CO_2 + T (Fig. 3c, Table 3). The total concentration of phenolic acids was 66% greater in females than in their male counterparts (Fig. 3c). In both sexes, the synthesis of phenolic acids was reduced under elevated temperature, while synthesis was increased under elevated CO_2 concentration (Fig. 4e, f).

Elevated temperature reduced the concentration of salireposide, a characteristic phenolic glucoside in *Salicaceae* bark, by 47% (Fig. 3d). GVA demonstrated that elevated CO_2 concentration affected the synthesis of salireposide differently in females than in males. In females, it reduced synthesis while in males, it accelerated synthesis (Fig. 4g, h). Under elevated temperature, there was a tendency of a dilution effect in females, while the concentration decreased in males as a result of decreased synthesis (Fig. 4g, h). In addition, elevated temperature reduced the concentration of lignan by 17%. Males had a 20% higher concentration of lignan than their female counterparts (Fig. 3e). The decrease caused by elevated temperature is mostly explained by dilution in both sexes (Fig. 4i, j).

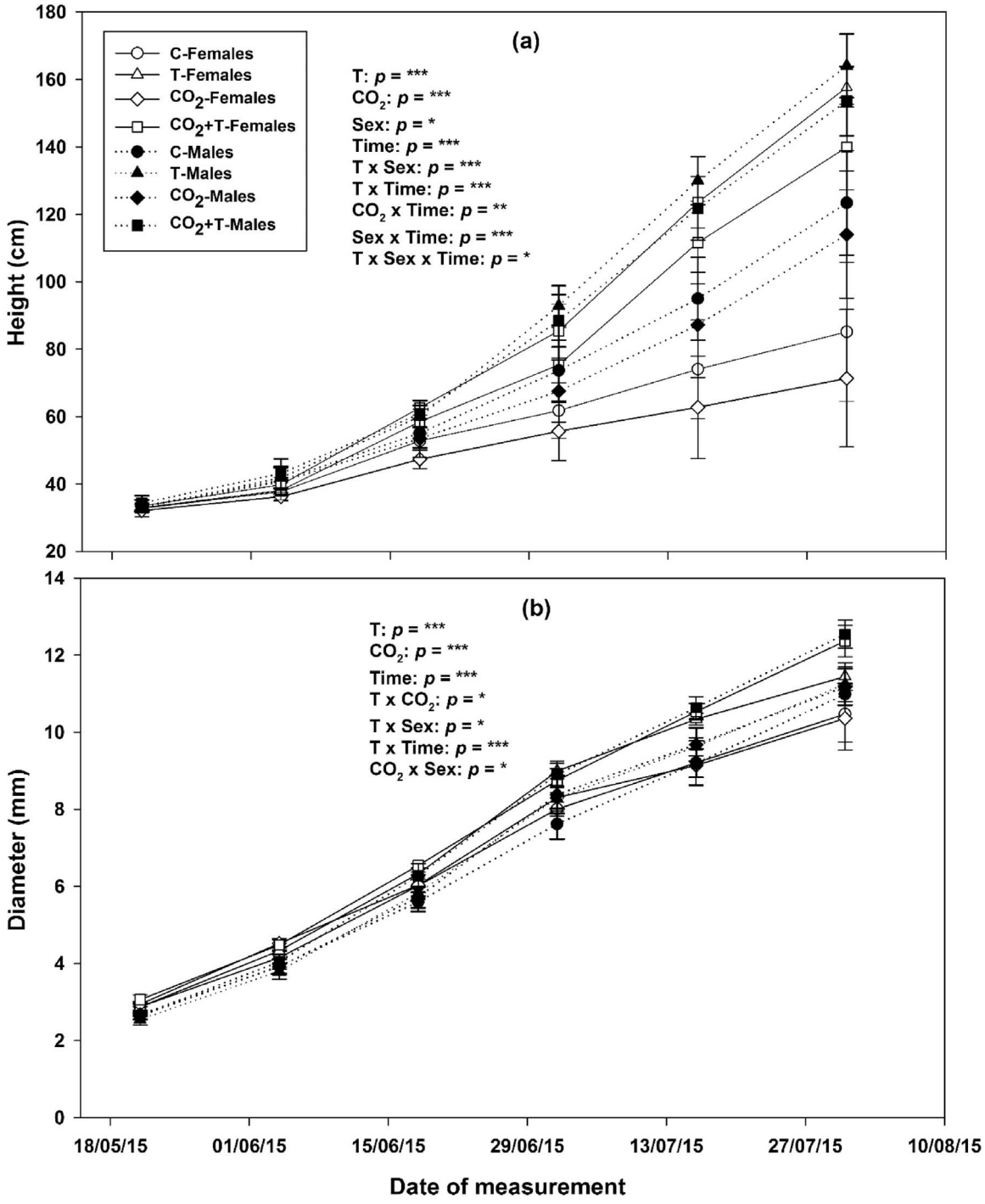

Fig. 1. Mean (± SE) height (a) and diameter (b) growth of *P. tremula* on different measurement dates under control (C), elevated CO_2 concentration (CO_2), elevated temperature (T) and CO_2 + T conditions. Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

4. Discussion

4.1. Effects of elevated temperature and CO_2 concentration on the growth of *P. tremula*

In our greenhouse experiment, the growth of *P. tremula* seedlings was more responsive to elevated temperature than to elevated CO_2 concentration in their first growing season. Elevated temperature stimulated all the measured growth parameters including height, diameter, leaf and stem biomass, which is in line with results from *Salix myrsinifolia* greenhouse (Veteli et al., 2002) and *S. myrsinifolia* and *P. tremula* field studies (Nybakken et al., 2012; Randriamanana et al., 2015). In the northern hemisphere, moderate temperature elevation usually increases the growth of trees, since growth is temperature limited in this region (Way and Oren, 2010). However, for a particular species, growth increment can depend on how close this species is already to its optimum growth temperature (Yamori et al., 2014). In this study, average (day-night) ambient and elevated temperatures were 16.1 °C and 17.6 °C, respectively during the experimental period. The strong growth responses to elevated temperature suggest that the optimum temperature limit for *P. tremula* has not yet been reached.

We assumed, like numerous earlier studies, that elevated CO_2 would

Table 2
F-values and degrees of freedom (df) obtained from the linear mixed model analysis of the effects of fixed factors and their combinations on the biomass of *P. tremula*. Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Arrows indicate the increase of the biomass parameters.

Factor	Leaf biomass			Stem biomass			Total biomass		
	F	*Numerator df*	*Denominator df*	*F*	*Numerator df*	*Denominator df*	*F*	*Numerator df*	*Denominator df*
T	75.259***↑	1	114	79.211***↑	1	114	82.811***↑	1	114
CO_2	0.996	1	114	0.035	1	114	0.321	1	114
Sex	0.735	1	6	0.468	1	6	0.616	1	6
T x CO_2	0.518	1	114	0.885	1	114	0.759	1	114
T x Sex	0.580	1	114	1.974	1	114	1.339	1	114
CO_2 x Sex	0.600	1	114	1.072	1	114	0.904	1	114
T x CO_2 x Sex	0.368	1	114	0.042	1	114	0.158	1	114

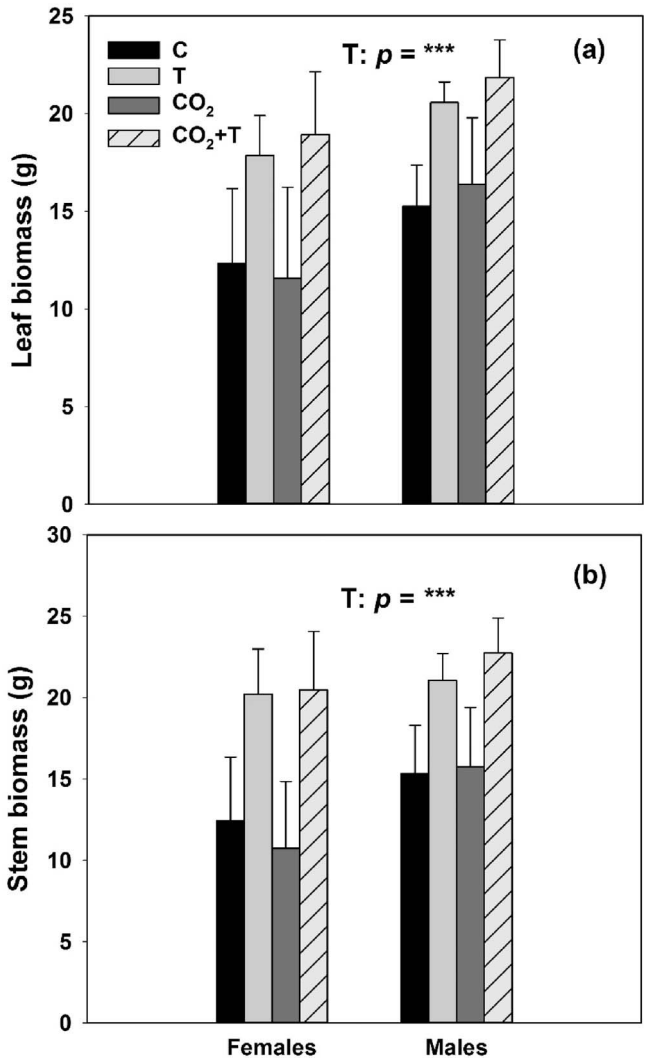

Fig. 2. Mean (± SE) leaf (a) and stem (b) biomass of *P. tremula* in dry weight under control (C), elevated CO_2 concentration (CO_2), elevated temperature (T) and CO_2 + T conditions. Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

increase the growth of *P. tremula* substantially (Norby et al., 1999; Veteli et al., 2002; Nybakken and Julkunen-Tiitto, 2013). However, elevated CO_2 led to a decrease in height and had no effects on diameter and biomass growth. Conroy et al. (1990), Yazaki et al. (2001) and Yazaki et al. (2004) also reported a reduction in height growth in *Pinus radiata, Larix sibirica and L. kaempferi* as a result of higher CO_2 concentration. In our study, CO_2 treated *P. tremula* had a shorter apical portion of the main stem and more branches compared to ones grown in ambient CO_2 (visual observation during the experiment). This weak apical dominance is probably due to the effects of elevated CO_2 on hormonal production or transportation in the shoot apex, as suggested by Conroy et al. (1990). Diameter growth was considerably higher under CO_2 + T than under any single treatment. Also, leaf and stem biomass tended to be greater under the combined effects of CO_2 and temperature when compared to other treatments. Comparatively higher leaf biomass under CO_2 + T indicates a greater cumulative leaf area, which in turn might facilitate higher assimilation and ultimately the increase in diameter and biomass growth. In fact, the greater production of assimilates under elevated CO_2 might be combined with the increased consumption of assimilates under elevated temperature, with less feedback inhibition of photosynthesis occurring under elevated CO_2 alone (Farrar and Williams, 1991; Usami et al., 2001).

In case of dioecious species, the degree of secondary sex differences evolve over time from pre-reproductive to reproductive stage (Bañuelos and Obeso, 2004; Montesinos et al., 2006). At sexual maturity, an increase in reproductive investments in females results in lower vegetative growth rate (Cipollini and Whigham, 1994; Obeso, 2002; Montesinos et al., 2006). In our study, intersexual difference in growth performance was only found in height growth, where males were significantly taller than the female individuals. In addition, males tended to have higher leaf and stem biomasses (Fig. 2). The absence of cost of reproduction in one-season old *P tremula* seedlings might explain the smaller intersexual differences in growth (Nicotra, 1999; Nybakken and Julkunen-Tiitto, 2013). Since males were partly growth-biased and elevated temperature stimulated the growth of *P. tremula* in this study, we therefore expected that males would benefit more from warming. However, height and diameter growth were greater for both sexes under elevated temperature, and the increments were considerably higher in females than in males. This may infer a variation in optimum growth temperature between females and males in *P. tremula*.

4.2. Effects of elevated temperature and CO_2concentration on phenolics in stem bark of P. tremula

In comparison to the case with foliar chemistry, very few studies have investigated secondary metabolites in stems of different tree species (Kuokkanen et al., 2001; Mattson et al., 2004; Lindroth et al., 2007; Nybakken and Julkunen-Tiitto, 2013; Randriamanana et al., 2014). Although the magnitude of phenolics accumulation varies between leaves and stems, the direction of accumulation in response to environmental factors may be similar in both organs (Cipollini et al., 1993; Chang et al., 2016). In our study, elevated temperature reduced the concentration of total phenolics in the stem bark of *P. tremula* since warming stimulated growth of the aspen. This trade-off between growth and phenolic chemistry as a response to elevated temperature supports earlier findings (Veteli et al., 2007; Randriamanana et al., 2015). In fact, the synthesis of shikimate-derived phenolic metabolites and plant growth depend on the common precursor, L-phenylalanine ammonia-lyase (PAL) (Matsuki, 1996; McDonald et al., 1999). In a period of

Table 3
F-values and degrees of freedom (df) obtained from the linear mixed model analysis of the effects of fixed factors and their combinations on the concentration of low molecular weight phenolics of *P. tremula*. Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Number before the compound name indicates the transformation applied ([1] = $\log_{10}$, [2] = sqrt). Arrows indicate the increase or decrease of the compounds. The letters "f" and "m" indicate the significant effect of a treatment on females and males, respectively.

Phenolic compound	Main effects			Interactions			
	T	CO_2	Sex	T x CO_2	T x Sex	CO_2 x Sex	T x CO2 x Sex
Parameters' *numerator df*	1	1	1	1	1	1	1
Diglucoside of salicyl alcohol	5.622*↑	1.759	0.343	0.807	9.466**	0.726	0.015
Denominator df	114	114	6	114	114	114	114
[1]Salicin	3.023	0.048	1.272	1.551	16.279***	0.352	1.298
Denominator df	114	114	6	114	114	114	114
Salicortin	99.745***↓	11.725**↑	0.534	0.467	2.846	1.941	0.077
Denominator df	114	114	6	114	114	114	114
[1]HCH-Salicortin	4.948*↑	1.647	14.782**f	0.119	0.047	0.009	0.259
Denominator df	100.988	100.373	6.138	100.421	100.988	100.373	100.421
Salicortin derivative	21.506***↓	10.169**↑	0.141	0.201	4.140*	0.286	0.471
Denominator df	113.070	113.070	6.036	113.070	113.070	113.070	113.070
Tremulacin	5.216*↓	4.142	0.152	0.679	0.040	0.575	0.153
Denominator df	114	114	6	114	114	114	114
Desalicortin	0.323	0.024	1.761	1.209	0.625	0.300	0.019
Denominator df	114	114	6	114	114	114	114
[1]Cinnamol salicortin	0.171	0.331	2.049	0.372	1.909	2.589	0.000
Denominator df	66.531	66.449	3.861	66.430	66.531	66.449	66.430
Cinnamoyl salicortin derivative 1	28.012***↓	5.653*↑	2.701	1.767	0.003	0.177	0.266
Denominator df	111.999	111.981	5.965	111.999	111.999	111.981	111.999
Cinnamoyl salicortin derivative 2	66.889***↓	7.870**↑	3.307	1.411	7.314	0.007	2.153
Denominator df	112.024	112.024	6.004	112.024	112.024	112.024	112.024
Cinnamoyl salicortin derivative 3	0.110	0.024	0.060	1.494	2.715	4.283*	0.048
Denominator df	112.012	112.012	5.963	112.012	112.012	112.012	112.012
Salicylates (total)	20.739***↓	10.166**↑	0.868	2.091	0.067	1.506	0.047
Denominator df	114	114	6	114	114	114	114
Gallocatechin	6.099*↑	0.842	0.371	0.946	0.036	0.859	2.428
Denominator df	83.987	84.740	6.615	84.829	83.987	84.740	84.829
(+)-Catechin	11.873***↓	9.150**↑	0.000	0.180	1.965	0.603	1.792
Denominator df	101.143	101.107	6.040	101.153	101.143	101.107	101.153
[2]Myricetin galactoside	5.795*↓	0.416	1.661	5.166	0.353	11.002***	0.423
Denominator df	111.989	112.001	5.978	112.001	111.989	112.001	112.001
Myricetin 3-glucoside	65.406***↓	2.246	2.884	1.152	36.212***	0.022	5.863*
Denominator df	101.028	101.046	5.894	100.960	101.028	101.046	100.960
Myrecetin derivative	1.730	0.048	0.381	5.395*	4.182*	4.744*	0.281
Denominator df	112.020	112.020	6.003	112.011	112.020	112.020	112.011
Flavonoids (total)	14.753***↓	2.176	1.054	4.416*	0.002	8.365**m	0.002
Denominator df	114	114	6	114	114	114	114
[2]*p*-OH-cinnamic acid glucoside	53.171***↓	14.940***↑	12.399*f	0.771	16.897***	0.030	1.295
Denominator df	114	114	6	114	114	114	114
p-OH-cinnamic acid derivative	62.793***↓	4.814*↑	3.894	0.174	5.554*	0.138	0.089
Denominator df	114	114	6	114	114	114	114
Chlorogenic acid derivative 1	14.830***↓	6.095*↑	9.660*f	0.026	15.420***	1.659	0.049
Denominator df	96.371	96.444	6.156	96.248	96.371	96.444	96.248
Chlorogenic acid derivative 2	75.167***↓	4.431*↑	2.614	0.003	2.485	0.001	0.158
Denominator df	111.988	112.032	5.988	111.988	111.988	112.032	111.988
Cinnamic acid derivative 1	2.662	3.784	0.000	1.712	0.358	0.043	1.057
Denominator df	105.985	105.985	5.909	105.985	105.985	105.985	105.985
[2]Phenolic acids (total)	102.558***↓	17.230***↑	5.964*f	0.040	10.341**f	0.311	0.609
Denominator df	114	114	6	114	114	114	114
Salireposide	44.031***↓	0.776	0.695	0.503	0.115	1.083	0.120
Denominator df	114	114	6	114	114	114	114
Lignan	15.629***↓	0.396	1.020	0.003	0.293	0.492	0.458
Denominator df	112.999	112.999	5.994	112.999	112.999	112.999	112.999
Low molecular weight phenolics (total)	54.676***↓	12.510**↑	0.954	2.672	0.007	2.648	0.025
Denominator df	114	114	6	114	114	114	114

higher growth, PAL activity is lower in plants, which in turn reduces the synthesis of secondary metabolites (Tuomi et al., 1988; McDonald et al., 1999). Although not measured here, a number of studies report an increase of the carbon/nitrogen ratio in plant tissues in response to elevated CO_2 concentration (Reichardt et al., 1991; Peñuelas and Estiarte, 1998; Tognetti and Johnson, 1999). A decrease in nitrogen concentration reduces the utilization of carbon for growth, and surplus carbon is then allocated to the synthesis of carbon-based phenolic metabolites (e.g. Bryant et al., 1983; Herms and Mattson, 1992). This may explain the increase in phenolic compounds in *P. tremula* under elevated CO_2 concentration, with identical growth between CO_2 treated and control plants.

Salicyl alcohol derived phenolic metabolites (salicylates) are prominent in *Populus* species (Tsai et al., 2006; Keefover-Ring et al., 2014; Randriamanana et al., 2015), although the ageing of the plant was shown to reduce the amount (Julkunen-Tiitto, 1986; Donaldson et al.,

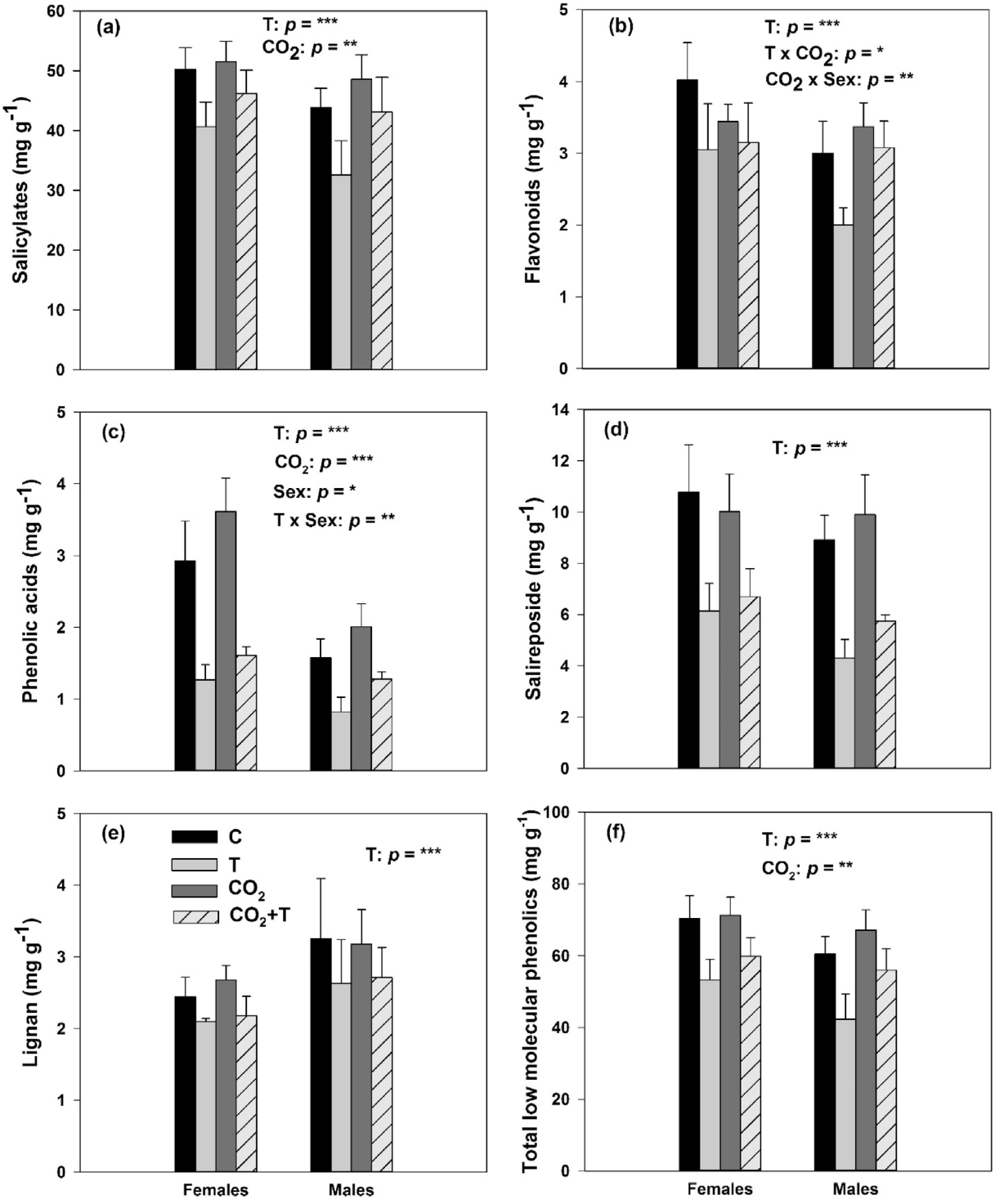

Fig. 3. Mean (± SE) concentration of salicylates (a), flavonoids (b), phenolic acids (c), salireposide (d), lignan (e) and total low molecular phenolics (f) in stem bark of *P. tremula* under control (C), elevated CO_2 concentration (CO_2), elevated temperature (T) and CO_2 + T conditions. Asterisks (*) denote the level of significance (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

2006). In our study, salicylates alone represented 74% of the total phenolics in bark tissues. Salicylates are important phenolic compounds in protecting plants against a wide range of herbivores, pathogens and abiotic stress (Tahvanainen et al., 1985; Hale et al., 2005; Lindroth and Clair, 2013). It has been reported that salicylates are relatively stable compounds in plant tissues having a very slow turnover rate (Ruuhola and Julkunen-Tiitto, 2000; Abreu et al., 2011; Keefover-Ring et al., 2014). In this study, many individual salicylates vary across the treatments, which resulted the overall decrease and increase in total concentrations of salicylates under elevated temperature and CO_2 concentration, respectively. This may be considered a potential fitness advantage in the face of future environmental changes. Tremulacin and salicortin, the most abundant compounds among the salicylates in bark tissues, are regarded as the most bioactive compounds in *Salicaceae species* and are known to reduce the growth and development of generalist herbivores (e.g. Tahvanainen et al., 1985; Chen et al., 2009; Lindroth and Clair, 2013). Although most of the salicylates were either reduced or did not change their concentrations, a diglucoside of

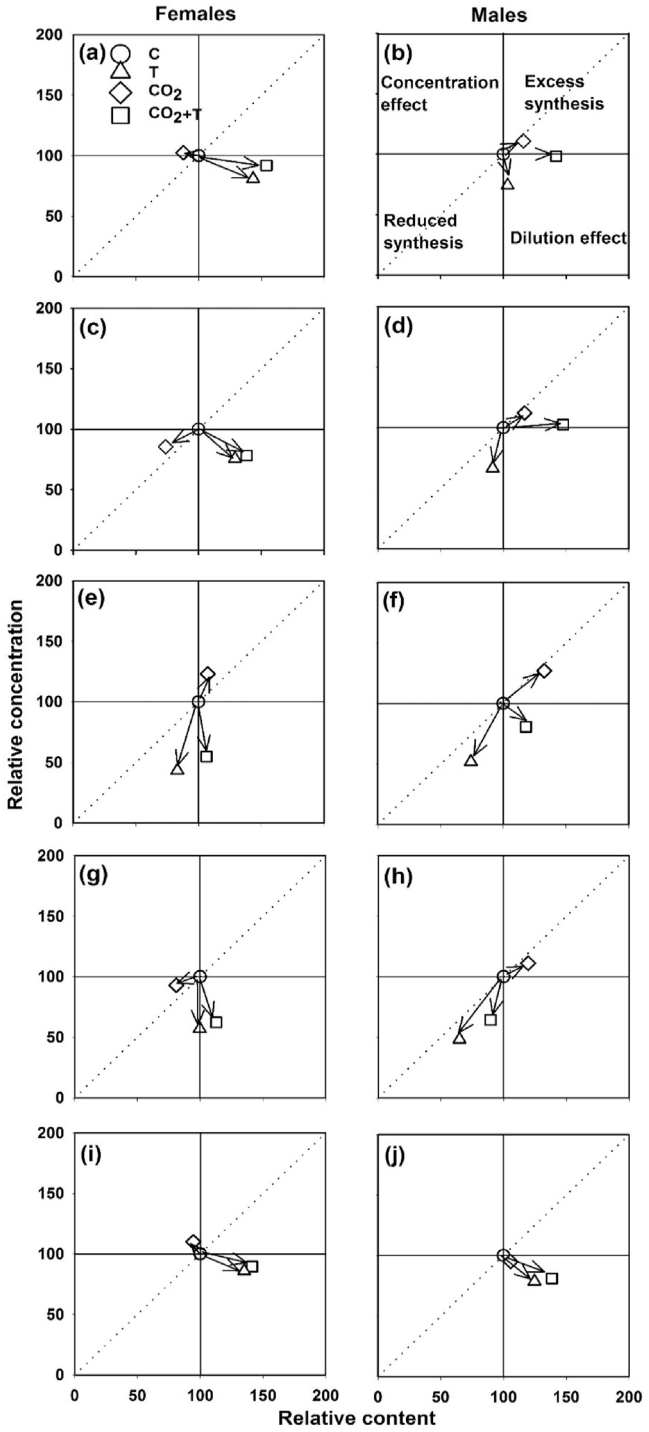

Fig. 4. Changes in salicylate (a, b), flavonoid (c, d), phenolic acid (e, f), salireposide (g, h) and lignan (i, j) concentrations (vertical axis, y) depending on their contents (horizontal axis, x) and stem biomass (diagonal axis, z) in stem bark of *P. tremula* under control (C), elevated CO_2 concentration (CO_2), elevated temperature (T) and CO_2 + T conditions.

salicylalcohol and HCH-salicortin increased in response to elevated temperature. HCH-salicortin, which is rarely found in *Populus* species (Tsai et al., 2006), is more toxic to insects than any related compounds as it contains two HCH (hydroxy-cyclohexen-on-oyl) functional groups (Lindroth et al., 1988; Randriamanana et al., 2014).

As compared to salicylates, the accumulation of flavonoids and phenolic acids was lower in the bark of *P. tremula*. Tuomi et al. (1988) stated that the synthesis of secondary metabolites depends on the ratio of carbon to nutrient, however certain interest of the plant may affect the allocation of carbon to particular substances. Since salicylates are the main compounds of defense in *Salicaceae* species (Boekler et al., 2011; Keefover-Ring et al., 2014), *P. tremula* might prioritize the accumulation of salicylates over accumulation of other secondary metabolites. Flavonoids and phenolic acids are reported to alleviate oxidative stress in plants (Sherwin and Farrant, 1998; Julkunen-Tiitto et al., 2015), and they have different antifungal, antimicrobial and antiherbivorous activities across a wide range of species (Panda and Khush, 1995; Tamagnone et al., 1998; Treutter, 2006), but their defensive strength remain to be studied in *Populus* species.

Studies on dioecious species have reported a greater allocation of carbon to phenolics in females than in males (Ågren et al., 1999 Nybakken and Julkunen-Tiitto, 2013). In this study, although statistically not significant, there was an indication of greater phenolics concentration in females as compared to males (Fig. 3). GVA revealed that both sexes responded similarly to elevated temperature regarding the accumulation of a particular group of compounds. Warming reduced salicylates and lignan concentrations in both sexes (dilution effect), while phenolic acids and salireposide caused a decrease not only in concentration, but also in content (reduced synthesis). Females followed the similar trend as males in carbon distribution between growth and phenolics under elevated temperature, which indicates that plants usually prioritize growth over the accumulation of secondary compounds under favorable condition (Tuomi et al., 1991) and that this might be irrespective of the gender. Similar to elevated temperature, elevated CO_2 concentration had no sex-specific effects on phenolics accumulation, except in the case of flavonoids, where elevated CO_2 concentration increased the flavonoids concentration in males (Table 2). GVA confirmed that flavonoids were increased in both concentration and content (excess synthesis) in CO_2-enriched males (Fig. 4d). While males had a relatively greater biomass than did their female counterparts, they allocated some extra carbon to the synthesis of expensive flavonoids along with the excess synthesis of cheaper salicylates and phenolic acids.

4.3. Conclusions

Our results indicate that *P. tremula* seedlings responded more to elevated temperature than to elevated CO_2 concentration in growth and phenolics accumulation, and that the direction of the responses was opposite. Elevated temperature increased the growth performance and decreased the accumulation of phenolics. On the other hand, elevated CO_2 decreased the height growth and increased the accumulation of phenolics. *P. tremula* seedlings tended to grow more under the combined effect, but elevated temperature counteracted elevated CO_2 concentration regarding phenolics accumulation. Although intersexual differences were not significant in the study, males tended to have higher growth, while females tended to have greater concentrations of phenolics in the stem bark. The smaller sexual differences were not strongly affected by elevated temperature and CO_2 concentration. In the future, with concomitant increases in CO_2 concentration and temperature, both sexes of *P. tremula* will probably grow more and accumulate lower levels of phenolics, but intersexual differences in growth and phenolics concentration may be more pronounced after sexual maturation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.envexpbot.2017.08.003.

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